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PURIFICATION AND PHYSIOCHEMICAL CHARACTERIZATION OF PYOMELANIN PIGMENT PRODUCED FROM LOCAL *PSEUDOMONAS AERUGINOSA* ISOLATES

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ABSTRACT

Pyomelanins are pigment of high molecular weight formed by oxidative polymerization of phenolic or indolic compounds and usually are dark brown or black produced by all organisms from microorganisms to human. At the moment pyomelanins are still enigmatic bio-pigments with structure and determination methods not clearly understood. Among biopolymers, pyomelanins are unique in many aspects. This study was designed to screen and characterizes pyomelanogenic local *pseudomonas aeruginosa* isolates with extraction and characterization of pyomelanin pigment. Eight pyomelanoenic isolates were identified out of 143 specimens from burns, urine and sputum were collected from Iraqi patients hospitalizedinto eleven hospitals which are located in Baghdad, Al-Anbar and Karbala provinces during the period of July 2013 to April 2014. All isolates were identified as *P. aeruginosa* according to morphological, cultural, biochemical characteristics, VITEK-2 and 16S rRNA. The results were showed that the isolate of sputum from cystic fibrosis (CF) was greatest in production of pyomelanin. Pyomelanogenic isolates were 5.6% which produced pyomelanin pigment on pyomelanin production medium. The black pigment was confirmed as pyomelanin based on the solubility in organic solvents, UV-Vis, FT-IR and XRD spectra analysis. This is the first report on screening of pyomelanogenic local *P. aeruginosa* isolates and characterization of the produced pyomelanin pigment.

KEYWORDS: *Pseudomonas aeruginos*a, pyomelanin pigment, physico-chemical characterization.

INTRODUCTION

Pseudomonas aeruginosa, a gram negative bacteria which is *main* opportunistic human pathogen that has been known for many years, this organism is extremely adaptable, has a high level of intrinsic antibiotic resistance, a wide range of virulence factors, and the ability to form biofilms.[1]

P. aeruginosa produces many types of soluble pigments such as pyocyanin and pyoverdin are the most common, other pigments produced are pyorubin (red), pyomelanin (dark brown/black) and pyoverdin (yellow/green) [2] . Pyomelanin production has been reported in *P. aeruginosa* isolates mainly from urinary tract infections and chronically infected Cystic Fibrosis (CF) patients.[2, 3, 4]

Pyomelanin is a group of negatively charged hydrophobic macromolecules formed by the enzymatic oxidation and subsequent polymerization of phenolic and/or indolic compounds, which are classified into four categories based on the intermediates of melanogenesis: eumelanin, pheomelanin, allomelanin and pyomelanin.[5, 6]

Pyomelanin, and other forms of melanin has been described to occur almost in every taxon of living organisms ranging from bacteria to human.[7, 8]

Pyomelanins are difficult to characterize because of their intractable chemical properties and the heterogeneity in their structural features. pyomelanin pigments, in fact, are composed of many different types of monomeric units that are connected through strong carbon-carbon bonds. In other words, the high insolubility and undefined chemical entities are the two main obstacles in complete characterization of pyomelanin pigment.[9]

In the recent years, there is a renewed consumer interest towards the use of natural dyes and pigments in food, cosmetic and pharmaceutical industries because synthetic pigments are perceived as undesirable and potentially harmful; some of them are considered as carcinogenic.[10] Therefore the study aimed to screen and characterizes pyomelanogenic local *P. aeruginosa* isolates with extraction and characterization of pyomelanin pigment.

MATERIALS AND METHODS

Isolates selection and pigment production

Pyomelanin-producing bacterium was isolated from 143 samples placed on L-tyrosine agar plates for several days. Eight samples were capable of producing black /brown pigment. They were picked out and identified as *P. aeruginosa*, based on their morphological and biochemical characteristics, VITEK-2 and 16S rRNA sequence.

In vitro melanization assays

In vitro melanization assays were performed to determine whether *P. aeruginosa* produces pyomelanin pigment from L-tyrosine. Cells were spread onto agar plates with or without L-tyrosine. The bacterial cells turned black colored within 3 days on agar plate containing L-tyrosine, but pigmentation was little observed in agar plates lacking L-tyrosine. The liquid medium containing L-tyrosine also turned black / brown after 2-3 days.[11]

Extraction and purification of pyomelanin pigment

Bacterial strains capable of producing high amounts of pyomelanin on pyomelanin production medium supplemented with L –tyrosine were isolated. Then the plates freezed and thawed to extract the pyomelanin pigment. The disrupted broth was acidified with 1 N HCl to pH 2 and allowed to stand for one week at room temperature. Then this suspension was boiled for 1 h to prevent the formation of melanoidins and then centrifuged at 8,000 g for 10 min.[11] The formed black pigment pellet was washed three times with 15 ml of 0.1 N HCl, and then washed with water. To this pellet, 10 ml of ethanol was added and the mixture was incubated in a boiling water bath for (10) min and kept at room temperature for 1 day. The pellet was washed with ethanol two times and then dried in air. The extracted pigment was pooled for use in subsequent analysis. The chemical analysis of pyomelanin pigment was carried out by the modified method of Fava et al.[11]

UV-Visible Spectroscopic Analysis of the Extracted Melanin

Different concentrations of purified pyomelanin were prepared using the initial concentrations of 100 mg/l in 0.1 N NaOH, and diluted to 1:1, 1:2 and 1:3. Each alkaline solution was scanned from 180 to 900 nm wavelengths. A 0.1 N NaOH was used as the blank.[12] The spectroscopic property of the melanin pigment obtained from *P. aeruginosa* was compared with synthetic pyomelanin[Sigma, santh Louis].

Fourier transform infrared (FT-IR) spectroscopy

For FT-IR spectrum, the pigment was further purified by acid hydrolysis with 5 ml of 3 N HCl in a sealed glass vial and kept for 2 h at 100°C.[13] The purified pigment was ground with infrared grade KBr (1: 10) and pressed into disks under vacuum using a Spectra Lab Pelletiser.The spectrum (400–4,000)cm^{-1} was recorded in a Shimadzu FTIR spectrophotometer.[14]

X-Ray Diffraction (XRD)

Pure pyomelanin powder was made into discs 1 cm in diameter and 1-2 mm thick. The pyomelanin pigment was scanned using a Shimadzu -LB6000 (Japan) X-ray diffractometer operating at a wavelength of 1.54056 Å with a step size of 0.02°and scanning rate of 2°/min X-ray beam at room temperature. Scattering intensity was recorded as a function of the scattering angle.[15]

RESULTS AND DISCUSSION

Extraction and purification of pyomelanin pigment

Eight pyomelanoenic samples of *Pseudomonas aeruginuosa* were isolated from 143 clinical samples from burns, urinary tract infections, respiratory tract infections and cystic fibrosis patients which picked from (11) hospitals in Iraq divided into three provinces, Baghdad, Al-Anbar and Karbala during the period of July 2013 to April 2014. These isolates were placed on L-tyrosine agar plates for several days Figure [1-A&B]. The colonies were capable of producing black / brown pigment which picked out and identified as *P. aeruginosa*, based on their morphological and biochemical characteristics as well as 16S rRNA sequence. Utilization of L-tyrosine by *P. aeruginosa* increased and caused more growth of the bacterium and pigment production. Maximum growth of the bacterium was observed on day 3 of incubation along with the maximum amount of pyomelanin production. The study of pyomelanin requires separation and purification of the pigment from its biological environment. The challenge is to develop procedures that do not modify the pigment during isolation, so the pigment obtained is reflective of its native form.[12,16,17]

The purification process of pyomelanin pigment derived from *P. aeruginosa* isolates is relatively easy and inexpensive, which needs only two steps. They are a simple acid precipitation followed by centrifugation. The precipitation step done by using 1N HCl below pH 2, which produced a brown flocculent precipitate in alkaline $FeCl_3$ (test for polyphenols) Figure.[2] These procedures are comparable to the pyomelanin purification protocols reported

in previous studies.[18, 19, 20] The purified pigment was positive to all main qualitative physico-chemical tests which were used to characterize pyomelanin pigment.

Physico- Chemical characteristics of pyomelanin pigment produced by local *P. aeruginosa* isolates

1. The solubility in organic solvents

The solubility of pyomelanin pigment in organic solvents is very limited. Pyomelanin was readily soluble in distilled water (both hot and cold), phenol, KOH and NaOH (100°C, 2 h), and insoluble in different organic solvents such as methanol, chloroform, acetone, hexane, ethyl acetate, benzene, methyl benzene, acetic acid, dichloromethane, acetonitrile and petroleum ether solvents. The solubility results were in agreement with Sajjan.[21]

2. UV-Vis Absorption spectra

UV absorption spectrum of pyomelanin exhibited maximum spectral peaks which were in the range of 235 -300nm for both extracted and standard synthetic pyomelanin pigments as shown in Figure (3&4). An increase in wavelength decreases the absorbance of melanin pigment progressively which is one of the most important criteria for the characterization of pyomelanin Schaeffer.[19] showed that the log of optical density of a pyomelanin solution, when plotted against wavelength, produces a linear curve with negative slopes. Such characteristic straight lines with negative slopes have been obtained for pyomelanin produced by some fungi.[15, 16, 12] The pigment produced by *Klebsiella* sp. GSK also gave a straight line with a negative slope indicating that it was pyomelanin.[15] When the pyomelanin was subjected to gradual dilutions, the absorbance decreased unevenly from the UV region to near the red region. This is due to the presence of much complex conjugated structure in the pyomelanin molecule. The similar reason that all spectra showed a strong UV absorption in the 200-300 nm region that can be attributed to the Л=> Л * and *n*=> Л* of the amino, carboxylic and aromatic moieties. Also, the presence of oxygen containing groups may contribute to the dark color of pigments. It is clear that pyomelanin efficiently and rapidly converts absorbed photon energy into heat.[17]

3. Fourier transform infrared (FT-IR) spectroscopy

FTIR spectroscopy technique was performed on extracted and standard synthetic pyomelanin pigment samples to obtain information about the chemical structures assigned to O-H and N-H stretching vibrations. The overlay of the FTIR spectra of synthetic and extracted pyomelanin from the supernatant cultures showed a high degree of similarity Figure (5). The

spectra depict a broad absorption at 3, 410 cm^{-1}, which is due to associated or polymeric OH groups. The peak observed at 2927 cm^{-1} suggested the presence of aliphatic CH bonding groups this is due to the asymmetric and symmetric stretching vibration of the melanin-alginate beads and the melanin pigment, which is mainly due to the presence of an -O-H group, usually H-bonded, and the presence of an aldehyde -C-H group. The analysis of the 1800-1400 cm^{-1} zone shows well defined maxima at 1651, 1516 and 1419 these maxima absorption were assigned to carbonyl stretching (C =O) and to aromatic rings, C = C/ C=N double bonds, conjugated with C =O and/or COO groups. While, 1455 cm^{-1} corresponding to (C-H) vibrations modes, 1400 cm^{-1} caused by oscillation of O- H groups of alcohols. The binding of metal ions to the melanin pigment causes a shift in the FT-IR vibration and depends on the percent adsorption, respectively. Extracted pigment showed high degree of resemblance in main absorption peaks which confirmed produced pigment is a pyomelanin. These results were in agreement with previous studies on melanin pigment.[18, 19, 20]

4. X-Ray Diffraction (XRD)

The XRD spectra of the extracted and standard pyomelanin pigment present a single broad diffraction peak on the 2h scale of 20°C in all the diffractograms forms (Fig. 6&7). Pyomelanin structures are uncertain due to the amorphous, heterogeneous and insoluble nature of these pigments.[21] Amorphous pyomelanins are not amenable to study by crystallography**.** The scattering of X-rays by crystalline structures produces sharp peaks in the diffraction spectrum that serve as a signature for the crystal that is analyzed. In contrast, the amorphous compound melanin produces broad features in a diffraction spectrum, known as non-Bragg features resulting in the absence of coherent scattering from regular.

and repeating structures as observed in crystals.[22,23,24] A consistent finding with all the samples is the produced pigment was positive to all main qualitative physico-chemical tests which were used to characterize pyomelanin pigment which was produced by local *Pseudomonas aeruginosa* isolates.

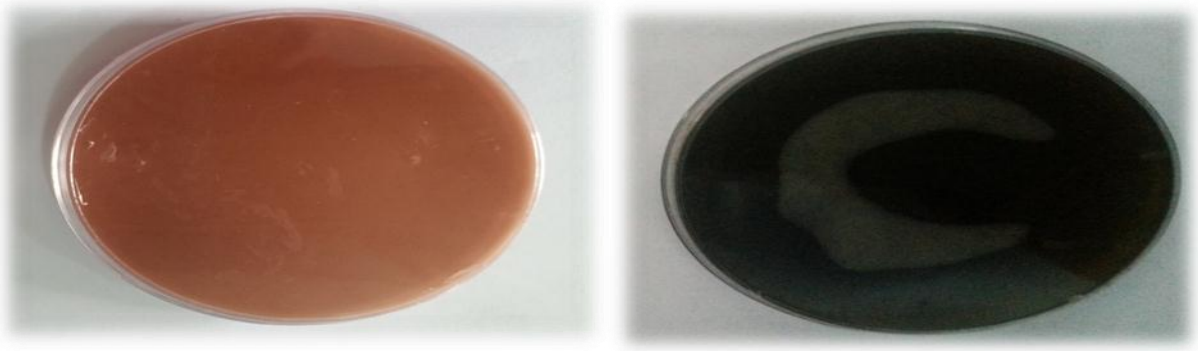

Figure (1): Pyomelanin pigment produced on pyomelanin production medium. A: after 24 h, B: after 72h. of incubation time at 37°C.

Figure (2): Extraction of pyomelanin pigment by using acid precipitation and centrifugation.

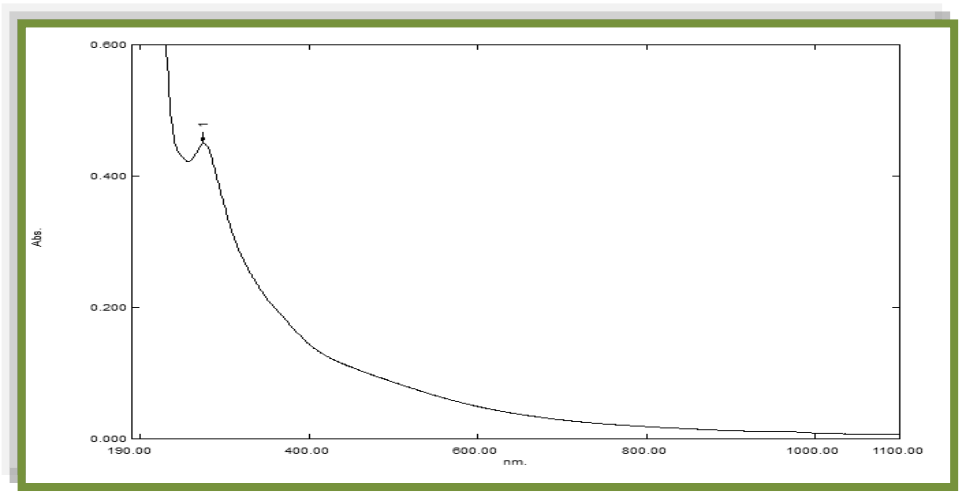

Figure (3): UV absorbance of synthetic pyomelanin pigment.

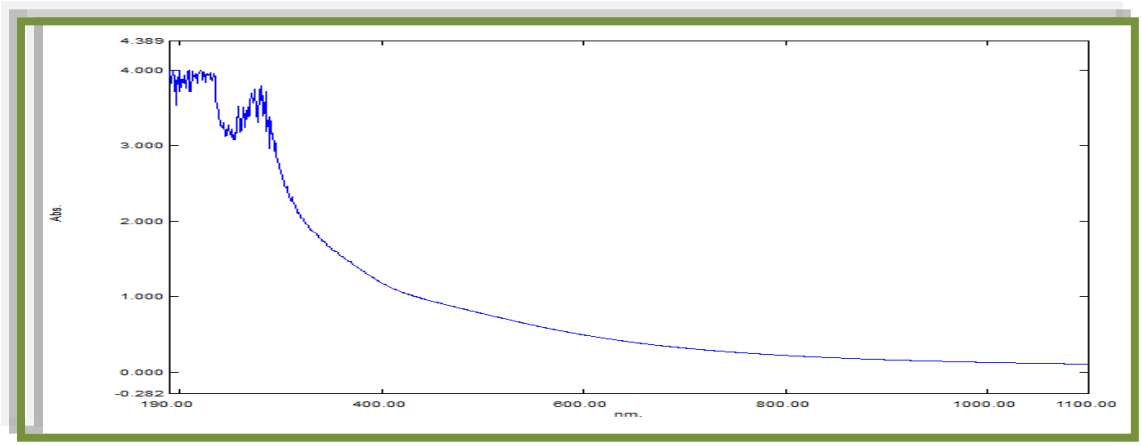

Figure (4): UV absorbance of extracted pyomelanin pigment.

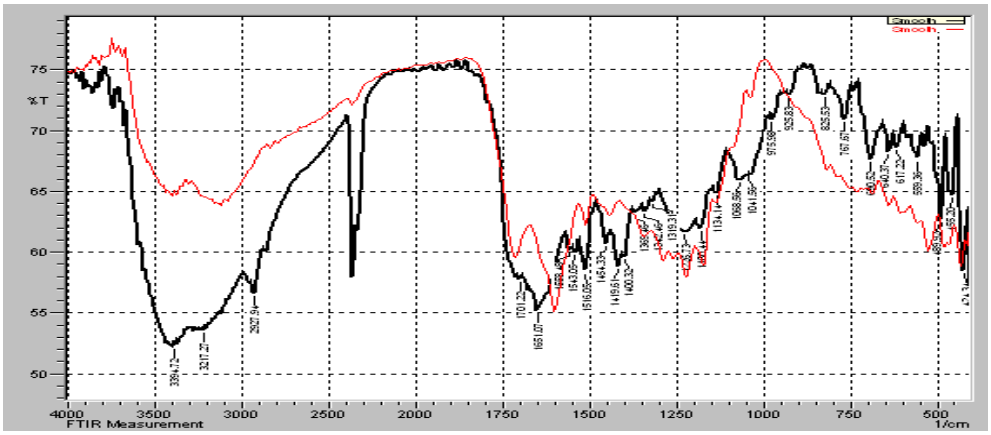

Figure (5): FTIR spectra of synthetic (red) and extracted pyomelanin pigment (black).

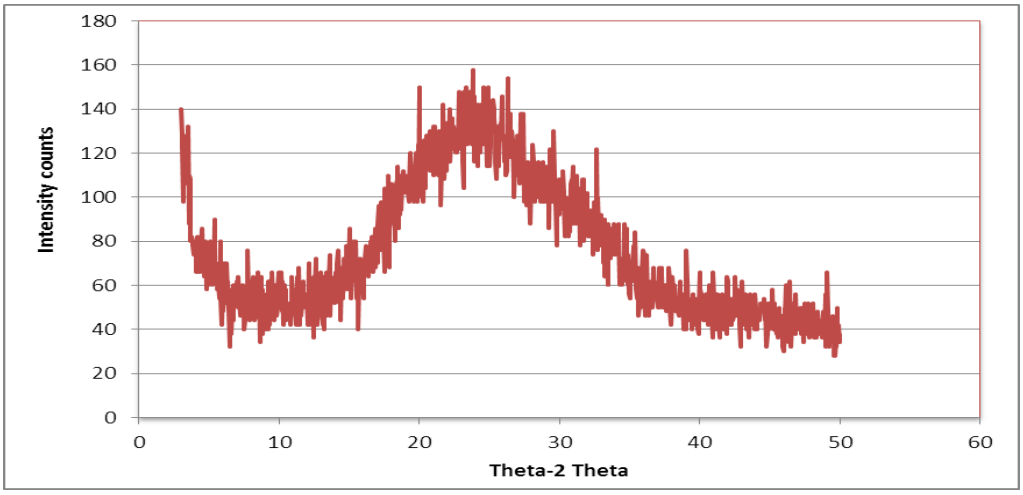

Figure (6): X-Ray Diffraction of synthetic pyomelanin pigment from local isolates.

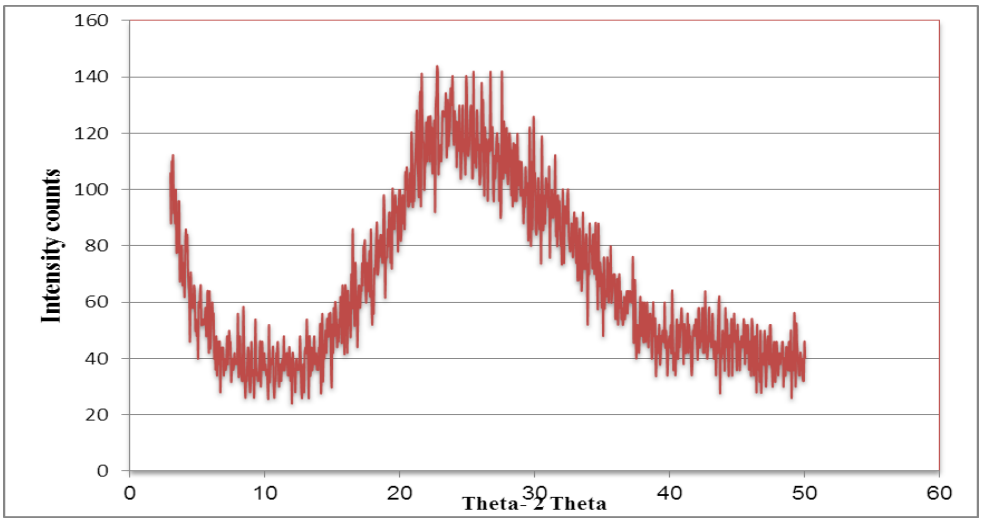

Figure (7): X-Ray Diffraction of extracted pyomelanin pigment from local isolates.

REFERENCES

1. Morrison J. and Wenzel p. Epidemiology of Infections Due to Pseudomonas aeruginosa. Clin Infect Dis., 1984; 6: 627-642.
2. Yabuuchi E, Ohyama A. Characterization of pyomelanin-producing strains of Pseudomonas aeruginosa. Int J Syst Bacteriol., 1972; 22: 53–64.
3. Rodriguez-Rojas A, Mena A, Martin S, Borrell N, Oliver A, Blazquez J. Inactivation of the hmgA gene of Pseudomonas aeruginosa leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection. Microbiology, 2009; 155: 1050–1057.
4. Hunter R, and Newman D. A putative ABC transporter, HatABCDE, in among molecular determinants of pyomelanin production in Pseudomonas aeruginosa. Bacteriol., 2010; 192: 5962–5971.
5. Riley P. Melanin. Int J Biochem Cell Biol., 1997; 29: 1235–1239.
6. White LP. Melanin: a naturally occurring cation exchange material. Nature., 1958; 182: 1427–1428.
7. Nosanchuk JD, Casadevall A. The contribution of melanin to microbial pathogenesis. Cell Microbiol., 2003; 5: 203-223.
8. Nosanchuk JD, Casadevall A. Impact of melanin on microbial virulence and clinicalresistance to antimicrobial compounds. Antimicrob Agents Ch, 2006; 50: 3519–3528.
9. Piattelli M., E. Fattorusso, Nicolaus R. A., and S. Magno The structure of melanins and melanogenesis V. Ustilago melanin. Tetrahedron.,1965; 21: 3229-3236.

10. Wang M., J. Li, M. Rangarajan, Y. Shao, E. J. La Voie, T. Huang and C. Ho. Antioxidative phenolic compounds from Sage (Salvia officinalis). J. Agr. Food Chem, 2006; 46: 4869-4873.
11. Fava F., Gioia D. D., and Merchetti L., Characterization of a pigment produced by Pseudomonas fluorescens during 3-chlorobenzoate co-metabolism. Chemosphere, 1993; 27: 825-835.
12. Wenlin Y. and Jeffrey O.D. Melanin biosynthesis by Frankia strain. CeI5. Physiol. Plant, 2007; 131: 180-190.
13. Bell AA. and Wheeler MH. Biosynthesis and functions of fungal melanins. Annu. Rev. Phytopath., 1986; 24: 411–451.
14. Ellis DH, Griffiths DA. The location and analysis of melanin in the cell walls of some soil fungi. Can J Microbiol, 1974; 20: 1386-1379.
15. Ravishankar J. P., V. Muruganandam, and T. S. Suryanarayanan. Isolation and characterization of melanin from a marine fungus. Botanica Marina., 1995; 38: 413-416.
16. Casadevall A., A. Nakouzi, P. R. Crippa, M. Eisner. Fungal melanins differ in planar stacking distances. PLoS ONE., 2012; 7: 30299.
17. Liu Y. T., M. J. Sui, D. D. Ji, I. H. Wu, C. Chou, and C. C. Chen. Protection from UV irradiation by melanin of mosquitocidal activity of Bt. var. israeliensis. Invertebr Pathol, 1993., 62: 131-136.
18. Schmaler-Ripcke J., Sugareva V., Gebhardt P., Winkler R., Kniemeyer O., Heinekamp T., and Brakhage A., A. Production of Pyomelanin, a Second Type of Melanin, via the Tyrosine Degradation Pathway in Aspergillus fumigatus Appl. Environ. Microbiol, 2009; 75: 493-513.
19. Schaeffer, P. A black mutant of Neurospora crassa: Mode of action of the mutant allele and action of light on melanogenesis. Arch. Biochem. Biophys., 1953; 47: 359-379.
20. Selvakumar P., S. Rajasekar, K. Periasamy, and N Raamn. Isolation and characterization of melanin pigment from Pleurotus cystidios (telomorph of Antromyocopsis macrocapa World J. Microbiol. Biotechnol., 2008; 24: 2125-2131.
21. Sajjan Shrishailnath S., Anjaneya O, Guruprasad B. Kulkarni, Anand S. Nayak, Pigment from Klebsiella sp. GSK. Korean J. Microbiol. Biotechnol., 2013; 41: 60–69.

22. Banerjee A., Supakar S. and Banerjee R. Melanin from the Nitrogen fixing Bacterium Azotobacter chroococcum: A Spectroscopic Characterization. PLoS One, 2014; 9: 1-7.
23. Turick C. E., L. S. Tisa, and J. F. Caccavo. Melanin production and use as a soluble electron shuttle for Fe (III) oxide reduction and as a terminal electron acceptor by Schewanella algae BrY. Appl. Environ. Microbiol., 2008; 68: 2436-2444.
24. Chávez-Béjar M., Balderas-Hernandez V. E., Gutiérrez- Alejandre. A., Martinez Francisco., B and o Gosset G. Metabolic engineering of Escherichia coli to optimize melanin synthesis from glucose. Microbial cell factories, 2013; 12: 1-12.
25. Microbial virulence and clinical resistance to antimicrobial compounds. Antimicrob Agents Ch, 2006; 50: 3519–3528.
26. Piattelli M., E. Fattorusso, R. A. Nicolaus, and S. Magno. (1965).The structure of melanins and melanogenesis V. Ustilago melanin. Tetrahedron, 1965; 21: 3229-3236.
27. Wenlin. Y., H. B. Stephen, and O. D. Jeffrey. Melanin biosynthesis by Frankia strain CeI5. Physiol. Plant, 2007; 131: 180-190.
28. Schaeffer, P. A black mutant of Neurospora crassa: Mode of action of the mutant allele and action of light on melanogenesis. Arch. Biochem. Biophys, 1953; 47: 359-379.
29. Bell AA. and Wheeler MH Biosynthesis and functions of fungal melanins. Annu Rev Phytopath, 1986; 24: 411–451.
30. Casadevall A., A. Nakouzi, P. R. Crippa, M. Eisner. Fungal melanins differ in planar stacking distances. PLoS ONE, 2012; 7: 30299.
31. Rodriguez-Rojas A, Mena A, Martin S, Borrell N, Oliver A, Blazquez J. Inactivation of the hmgA gene ofPseudomonas aeruginosa leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection. Microbiology, 2009; 155: 1050–1057.
32. Hunter R, Newman D. A putative ABC transporter, HatABCDE, in among molecular determinants of pyomelanin production in Pseudomonas aeruginosa. J Bacteriol, 2010; 192: 5962–5971.
33. Ravishankar J. P., V. Muruganandam, and T. S. Suryanarayanan. Isolation and characterization of melanin from a marine fungus. Botanica Marina, 1995; 38: 413-416.
34. Selvakumar P., S. Rajasekar, K. Periasamy, and N Raaman. Isolation and characterization of melanin pigment from Pleurotus cystidios (telomorph of Antromyocopsis macrocapa.(World J. Microbiol. Biotechnol, 2008; 24: 2125-2131.